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Regulation of muscarinic acetylcholine receptor sensttivity and density was examined upon pre-exposure to muscarinic receptor agonists. Pretreatment of neuronal cells in culture resulted in a significant decrease in the response to subsequent exposure to agonists, in terms of cyclic GMP formation and the increase in phosphoinositide hydrolysis. This treatment also resulted in a decrease in the concentration of cell surface muscarinic receptors. This decrease in receptors was not accompanied by a change in receptor affinity for nonselective muscarinic receptor ligands. However, it resulted in a selective decrease in the relative densities of M_1 muscarinic receptors which have a high affinity for the selective antagonist pirenzepine. Activation of protein kinase C by phorbol esters also resulted in decreasing the responsiveness of muscarinic receptors. However, there was no change in receptor density. Keywords: Muscarinic antagonists, [illegible], Quinuclidinyl benzilate (QNB)

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Desensitization and Down-Regulation of Muscarinic Acetylcholine Receptors

FINAL REPORT

by

Esam E. El-Fakahany, Ph.D.

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Contract #DAAG29-85-K-0123 (21760-LS)

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assay medium. For this purpose, complete saturation isotherms of [^{3}H]N-methylscopolamine were performed in control and desensitized cells (1 mM carbamylcholine, 30 min at 37°C), where it was found that preincubation with the agonist results in a 50% reduction in the B_{max} without any effect on the ligand affinity, ruling out the possibility of such an artifact.

To elucidate further that carbamylcholine-induced reduction in [^{3}H]N-methylscopolamine binding is a muscarinic receptor-mediated phenomenon, the effect of atropine on this process was studied. Pretreatment of cells with 10 nM atropine for 30 min at 37°C was found to retard subsequent carbamylcholine-induced desensitization drastically, as compared to naive cells not pretreated with the antagonist. On the other hand, atropine by itself did not cause any significant alterations in subsequent [^{3}H]N-methylscopolamine binding, suggesting that receptor activation is a necessary prerequisite for this phenomenon. In addition, agonist-induce reduction in [^{3}H]N-methylscopolamine binding took place only at temperatures above 20°C and increased with increasing the incubation temperature. The activation energy of the process was calculated to be 18 Kcal/mole, a value which is similar to that of desensitization of cyclic GMP formation in the same cells. Rapid and complete recovery of agonist-mediated receptor down-regulation took place within 30 min at 37°C, while no significant recovery occurred at 15°C.

In summary, cell surface muscarinic receptors in mouse neuroblastoma cells are subject to rapid regulation upon exposure to agonists. This phenomenon shares several characteristics with agonist-induced desensitization of cyclic GMP synthesis, including time course, dependence on agonist concentration, temperature sensitivity and reversibility. These observations suggest a possible implication of this regulation of surface receptors in desensitization of receptor function.

These studies of the regulation of muscarinic receptor density by exposure to agonists in mouse neuroblastoma cells were replicated in intact brain cell aggregates dissociated from adult rat brains. Initial experiments were concerned with a detailed investigation of the binding characteristics of [^{3}H]N-methylscopolamine in this system. It was found that the specific binding of 1 nM [^{3}H]N-metylscopolamine to muscarinic receptor increased linearly with increasing the amount of cell protein in the assay, up to 400 μg of protein. When the concentration of [^{3}H]N-methylscopolamine was varied over the range of 0.01 - 2 nM, the specific binding of the ligand was saturable, with very low level (< 5%) of nonspecific binding. Analysis of the saturation isotherms resulted in linear Scatchard plots with a B_{max} of 450 fmoles/mg protein and a K_D of 0.17 nM, suggesting that [^{3}H]N-methylscopolamine binds to a single homogeneous population of muscarinic receptors in rat brain cell aggregates, with no evidence of cooperativity, as indicated by a Hill coefficient of unity.

Displacement of the specific binding of 0.2 nM [^{3}H]N-methylscopolamine in intact brain cell aggregates by the muscarinic antagonists scopolamine, atropine or QNB resulted in steep displacement curves which could be analyzed sa-

tisfactorily according to a single binding site. On the other hand, the muscarinic agonists oxotremorine and carbamylcholine exhibited very shallow displacement curves which could be resolved into high- and low-affinity states by iterative nonlinear regression analysis.

These studies have suggested that dissociated adult rat brain cells may provide a useful and physiologically relevant model to study the regulation of muscarinic receptor binding, and probably function, in vitro. This technique offers an easy method to obtain intact differentiated brain cells with minimal diffusion barriers.

Preincubation of rat brain cell aggregates with carbamylcholine resulted in a time-dependent decrease in subsequent [^{3}H]N-methylscopolamine specific binding, an effect which reached a steady state in 3 hrs at 37°C. This effect of carbamylcholine was dependent on the concentration of the agonist in the incubation medium, with a half-maximal response occurring at 10 μM. Similar to the findings in neuroblastoma cells, this agonist-induced down-regulation of [^{3}H]N-methylscopolamine binding was due to a reduction in the B_{max} with no significant change in the K_D. In addition, this phenomenon was absent when incubation with the agonist was performed at 15°C or in the presence of 100 nM atropine. Furthermore, the time course a disappearance of [^{3}H]N-methylscopolamine binding upon incubation with 1 mM carbamylcholine at 37°C was faster than that of [^{3}H]quinuclidinyl benzilate binding. These results suggested that desensitizing conditions are accompanied by a selective reduction in the concentration of surface muscarinic detected by [^{3}H]N-methylscopolamine. Another possible explanation is that these conditions transform the receptor into a conformation which can recognize [^{3}H]quinuclidinyl benzilate but not [^{3}H]N-methylscopolamine.

The effects of brief incubation with carbamylcholine on muscarinic receptor subtypes were investigated in mouse neuroblastoma cells (clone N1E-115). This treatment demonstrated that the muscarinic receptors in this neuronal clone can be divided into two types; one which is readily susceptible to regulation by receptor agonists, while the other is resistant in this regard. In control cells, both pirenzepine and carbamylcholine interacted with high- and low-affinity subsets of muscarinic receptors. Computer-assisted analysis of the competition between pirenzepine and carbamylcholine with [^{3}H]N-methylscopolamine showed that the receptor sites remaining upon desensitization are composed mainly of pirenzepine low-affinity and agonist high-affinity binding sites. Furthermore, there was an excellent correlation between the ability of various muscarinic receptor agonists to induce a decrease in consequent [^{3}H]N-methylscopolamine binding and their efficacy in stimulating cyclic GMP synthesis in these cells. Thus, only the agonists which are knows to recognize the receptor's low-affinity conformation in order to elicit increases in cyclic GMP levels were capable of diminishing ligand binding. Taken together, these results suggest that the receptor population which is sensitive to regulation by agonists includes both the pirenzepine high-affinity and the agonist-low affinity receptor binding states. In addition, the sensitivity of these recep-

tor subsets to rapid regulation by agonists further implicates their involvement in desensitization of muscarinic receptor-mediated cyclic GMP formation.

Due to the selective decrease in the high-affinity sites of [^{3}H]N-methylscopolamine and [^{3}H]pirenzepine in neuroblastoma cells upon short-term desensitization, it was important to study the nature of the differences in binding of theses two ligands and the binding of [^{3}H]quinuclidinyl benzilate to muscarinic receptors. These experiments were planned to help to explain why desensitization preferentially affects the binding of some muscarinic lignads but not others. Rat brain homogenates were used for this purpose due to the abundance of muscarinic receptors in this preparation relative to that in neuroblastoma cells.

The properties of the specific binding of the muscarinic receptor ligands [^{3}H]quinuclidinyl benzilate and [^{3}H]-methylscopolamine in rat brain were compared. The specific binding of both ligands was affected equally by heat, phospholipase A_2 and trypsin. [^{3}H]N-methylscopolamine labeled only a fraction of the total muscarinic receptors recognized by [^{3}H]quinuclidinyl benzilate in different brain areas and in the heart. Evidence has been found that [^{3}H]N-methylscopolamine, in fact, binds to a subpopulation of [^{3}H]quinuclidinyl benzilate binding sites. The distribution of the high-affinity binding sites of [^{3}H]N-methylscopolamine did not show a different tissue dependence as compared to the total receptor population, and did not parallel the distribution of the pirenzepine-sensitive M_1 receptor subtype. Similarly, the affinity of both [^{3}H]quinuclidinly benzilate and [^{3}H]N-methylscopolamine varied from one tissue to another by a maximum of two fold. Although (-)quinuclidinly benzilate competed for the specific binding of [^{3}H]quinuclidinly benzilate in different tissues according to the law of mass-action, N-methylscopolamine showed an anomalous interaction with two binding sites. The low-affinity binding sites of N-methylscopolamine showed saturability of [^{3}H]quinuclidinly benzilate binding and stereoselectivity. When the binding characteristics of these N-methylscopolamine-inaccessible binding sites of [^{3}H]quinuclidinly benzilate in the brain were investigated further, it was found that N-methylscopolamine bound exclusively with a single low affinity, while pirenzepine still interacted with two receptor populations incorporated in these sites. It is concluded from several lines of evidence that the heterogeneity of binding of N-methylscopolamine to muscarinic receptors does not represent an interaction with the muscarinic M_1 and M_2 receptor subtypes defined by pirenzepine. Thus, the unique binding profile of pirenzepine to muscarinic receptors cannot be explained merely on the basis of its hydrophilic nature.

We also studied the details of the interaction of the nonclassical muscarinic receptor antagonist pirenzepine with [^{3}H]quinuclidinly benzilate binding sites in rat brain homogenates. Pirenzepine showed biphasic competition curves with a Hill coefficient lower that than unity, and these curves were better described according to a two-site receptor model. The affinities and the relative preponderance of these sites were constant at different ligand concentrations, in accordance with a competitive type of interaction. Similar-

ly, pirenzepine did not influence the rate of dissociation of the [^{3}H]quinuclidinly benzilate-receptor complex even at relatively high concentrations. However, although low concentrations of pirenzepine decreased the affinity of [^{3}H]quinuclidinyl benzilate for the receptor without affecting the density of the binding sites, higher concentrations of the antagonist decreased the receptor number in a reversible fashion. Schild plots of these data indicated an apparent deviation from simple competition in this experimental design, an observation which can be attributed to the selectivity of pirenzepine for different receptor subtypes. Furthermore, pirenzepine, at concentrations high enough to saturate both its high and low-affinity sites protected [^{3}H]quinuclidinly benzilate binding sites in the brain against irreversible alkylation by propylbenzilylcholine mustard. Therefore, these data support a competitive nature of interaction of pirenzepine with brain muscarinic receptors.

Muscarinic receptors in mouse neuroblastoma N1E-115 cells, and in other tissues, mediated an increase in phosphoinositide hydrolysis. Diacylglycerol is one of the important products of this reaction, and it has been proposed to be the endogenous activator of protein Kinase C. Due to the involvement of protein Kinase C in the regulation of several neurotransmitter receptors, we investigated the effects of activation of this Kinase by phorbol esters on the sensitivity of muscarinic receptors in mouse neuroblastoma cells. Incubation of cells with phorbol-12-myristate-13-acetate (PMA) resulted in a time and concentration-dependent decrease in muscarinic receptor-mediated cyclic GMP synthesis. On the other hand, PMA had no effect on cyclic GMP formation induced by direct activation of guanylate cyclase by sodium azide. Although PMA did not compete directly for muscarinic receptors, nor did it decrease [^{3}H]N-methylscopolamine binding under conditions required to suppress cyclic GMP responses, PMA treatment resulted in the loss of pirenzepine high-affinity sites.

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